Microbial Enhanced Oil Recovery

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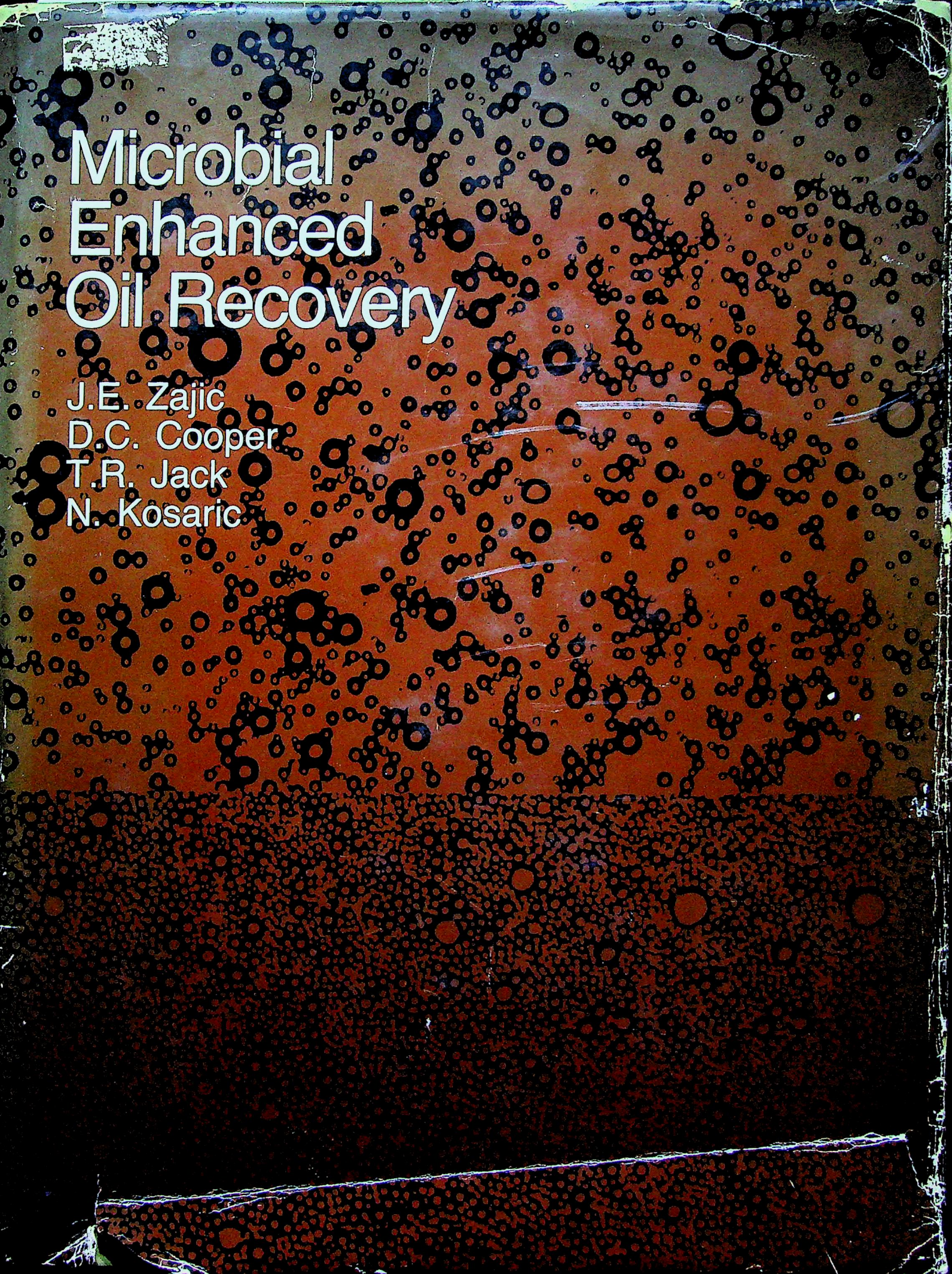

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PennWell Books
PennWell Publishing Company
Tulsa, Oklahoma

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PennWell Publishing Company
1421 South Sheridan Road/P.O. Box 1260
Tulsa, Oklahoma 74101

Library of Congress cataloging in publication data
Main entry under title:

Microbial enhanced oil recovery.
Bibliography
1. Secondary recovery of oil—Congresses.
2. Industrial microbiology—Congresses. I. Zajic,
J. E. (James E.), 1928-
TN871.M446 1983 622′.3382 82-18049
ISBN 0-87814-212-6

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Printed in the United States of America

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The book is dedicated to Hemisphere Licensing Corp., its subsidiary, Petroleum Sciences Inc. of Houston, and to all of those researchers who are advancing the frontiers of technology in microbial enhanced oil recovery.

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17 BACTERIAL ADHERENCE TO HYDROCARBONS

M. Rosenberg, D.L. Gutnick, and E. Rosenberg

Tel Aviv University

The importance of cell adherence in growth physiology of many bacterial species is well documented. For example, *Caulobacter* cells divide only after they attach to solid surfaces.[1] The initial step in the life cycle of *Bdellovibrio* is attachment to Gram-negative bacteria.[2,3] Specific cell-to-cell interactions pervade the entire life cycle of the myxobacteria.[4,5] Adhesion is a prerequisite to the successful colonization by microorganisms of animals and plants.[6,7] Microbial adhesion to surfaces has been the subject of several recent reviews.[6,7,8,9,10]

In cases where the carbon or energy source is a water-insoluble material, such as cellulose or chitin, bacterial adhesion can facilitate growth.[10,11,12] But cell contact is not an absolute requirement because extracellular enzymes can degrade these polymers into water-soluble substrates. However, growth of microorganisms on hydrocarbons presents a special problem since hydrocarbons are immiscible with water and their breakdown cannot occur extracellularly. The first step in aromatic or aliphatic hydrocarbon degradation is introduction of molecular oxygen into the molecules by cell-associated enzymes.[13,14,15]

Two general types of hydrocarbon-cell interactions have been postulated, depending upon the state and size of the oil droplets relative to the size of the microbial cells: (*i*) adherence of cells to large oil droplets and (*ii*) pseudosolubilization involving the cellular assimilation of emulsified small hydrocarbon droplets.[16] The relative contribution of these two types of interactions to the growth of bacteria on hydrocarbons has been difficult to ascertain experimentally. The frequently observed relationship between hydrocarbon-aqueous interfacial area and growth rate can be used to support either hypothesis.[17]

Adherence and growth of microorganisms on hydrocarbons is one example of a wide spectrum of phenomena in which bacterial hydrophobicity and adherence to hydrophobic surfaces are involved. The hydrophobic nature of the outermost bacterial surface has been cited as a factor in partitioning microorganisms at the air-water interface;[18,19] in the adherence of bacteria to nonwettable plastic surfaces,[20,21,22,23] and in the attachment of bacteria to phagocytes and other mammalian cells.[24,25,26,27,28]

A rapid and quantitative method for measuring bacterial adherence to hydrocarbons was described previously.[29,30] The present report deals with the use of this technique in (*i*) examining the cell surface hydrophobicity of various bacterial species, some of which do not metabolize hydrocarbons, and (*ii*) selecting and isolating nonadherent mutants. The isolation of a nonadherent mutant of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 has enabled us to examine the role of adherence in bacterial growth on hexadecane.

MATERIALS AND METHODS

Bacterial Strains. Tables 1 and 2 contain a list of bacteria used in this study. *Acinetobacter calcoaceticus* MR-481 is a nonadhering derivative of strain RAG-1.

Media and Growth Conditions. For measuring adherence to test hydrocarbons or preparing inocula for growth experiments, 0.1 ml of bacteria from an overnight culture was inoculated into 50 ml of growth medium and was incubated in a New Brunswick model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested at the end of the

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Table 1. Adherence of bacteria to hexadecane.

Strain	*Source*	*Growth Medium*[a]	*Adherence to Hexadecane (%)*[b]
Acinetobacter calcoaceticus RAG-1	this laboratory	BHI	97
Bacillus cereus	local isolate	NB	1
Bacillus subtilis 168	E.Z. Ron	NB	0
Enterobacter aerogenes CDC 659/66	E.Z. Ron	NB	9
Escherichia coli B	E.Z. Ron	NB	0
Escherichia coli K12	E.Z. Ron	NB	2
Escherichia coli J5 (rough)	E.Z. Ron	NB	11
Micrococcus lysodeikticus	I. Friedberg (ATCC 4698)	NB	0
Myxococcus xanthus	this laboratory	CT	35
Proteus mirabilis S1959	S. Rottem	NB	0
Pseudomonas aeruginosa PAS279	J. Shapiro	NB	0
Serratia marcescens	R. Zack	NB	96
Staphlococcus albus	R. Zack	NB	0
Staphlococcus aureus	R. Zack	NB	91
Streptococcus pyogenes M5	I. Ofek	BHI	84

[a] Cells were harvested at the end of the exponential phase

[b] The standard adherence assay was performed with 0.2 ml hexadecane.

exponential phase, washed twice, and resuspended in PUM buffer (22.2 g $K_2HPO_4 \cdot 3H_3O$; 7.26 g KH_2PO_4; 0.8 g urea; 0.2 g $MgSO_4 \cdot 7H_2O$; and distilled water to 1,000 ml, pH 7.1). Growth media were BHI, brain heart infusion broth (Difco); hexadecane medium, PUM buffer supplemented with 0.2% (v/v) hexadecane (olefin free, 99% purity, Fluka, Switzerland); P-hexadecane medium, 5 g NH_4NO_3, 2.5 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 2 ml hexadecane, 50 mg threonine, and tap water to 1,000 ml; and NB, nutrient broth (Difco) supplemented with 0.5% NaCl. CT medium contained 1% casitone (Difco) and 0.2% $MgSO_4 \cdot 7H_2O$[31].

In the growth experiments, washed cells of RAG-1 and MR-481 were inoculated into acid-washed Klett tubes (14-mm diameter) containing 4.2 ml of an aqueous medium to give an initial turbidity of 5 Klett units. The acetate growth medium consisted of PUM buffer supplemented with 0.2% sodium acetate. Hexadecane growth medium consisted of 0.5 ml hexadecane (olefin free, 99% purity, Fluka, Switzerland) layered onto 4.2 ml of PUM buffer. The test tubes were incubated upright in a New Brunswick gyrotory water bath shaker (Model G-76) at 330 rpm at 30°C. Turbidity was monitored directly in the growth tubes by means of a Klett-Summerson colorimeter fitted with a green filter.

Adherence to Hydrocarbons. The technique for measuring adherence of bacteria to hydrocarbons has been

Table 2. Adherence of *Acinetobacter* strains to hexadecane.

Strain Designation	*Source*	*Growth on Hexadecane*[a]	*Adherence to Hexadecane (%)*[b]
RAG-1 (ATCC 13012)	this laboratory	+	82
HO1-N	W.R. Finnerty	+	99
BD4	E. Juni	+	0
BD413	E. Juni	+	4
B5W str 71 (ATCC 15149)	K. Bryn	+	84
CDC 7827 (ATCC 17959)	K. Bryn	+	86
B5W (ATCC 17903)	K. Bryn	−	0
ATCC 17906	K. Bryn	−	52
ATCC 17977	K. Bryn	−	7
ATCC 17985	K. Bryn	−	41

[a] Growth was examined on PUM agar in the presence of hexadecane vapors.

[b] Adherence was measured on cells grown to stationary phase on BHI broth; the standard adherence assay was performed with 0.2 ml hexadecane.

described previously.[32] Cells were washed twice and were resuspended in PUM buffer to an initial A_{400} of approximately 1.5. Various volumes of the test hydrocarbons (n-hexadecane, n-octane, or p-xylene) were added to round-bottom, acid-washed test tubes (10-mm diameter) containing 1.2 ml of washed-cell suspension, and the phases were vortexed uniformly for 120 sec. After allowing 15 min for the phases to separate, the absorbance of the lower aqueous phase of each tube was measured at 400 nm (Gilford model 240 spectrophotometer, 1-cm light path). Adherence was calculated as the percentage loss in absorbance relative to that of the initial cell suspension.

Isolation of the Nonadherent Mutant MR-481. A spontaneous mutant of RAG-1 that is deficient in its ability to adhere to hydrocarbons was isolated by the following technique: RAG-1 cells were inoculated into 50 ml of a nutrient broth medium (NB) and were incubated in a New Brunswick Model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested in stationary phase following 18 hr growth, washed twice, and resuspended in PUM buffer to an A_{400} of approximately 1.5. Two ml of n-octane were added to 5 ml of the cell suspension, and the phases were vortexed for 5 min. Following phase separation the lower aqueous phase was tranferred to a second test tube. Octane was again added, and the phases were mixed as described previously. This extraction process was continued until cells in the lower aqueous phase were too few to be visible under microscopic examination. The final aqueous phase served as the inoculum for a second nutrient broth culture that was grown to stationary phase as previously described. Cells were again harvested, washed, and extracted with octane, and the final aqueous phase was then inoculated into a third nutrient broth culture. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even following several extraction attempts with octane. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even following several extraction attempts with octane. The final aqueous phase was plated on nutrient agar. Three individual colony types were isolated and tested for adherence to hydrocarbons. One clone of cells, termed MR-481, exhibited no significant affinity towards the test hydrocarbons and was chosen for further study.

Emulsan Preparation. The general method for preparing the extracellular emulsifying agent of *A. calcoaceticus* RAG-1, referred to as emulsan, was described previously.[33] The particular preparation used in this study was further purified by precipitating an aqueous solution of emulsan with cetyltrimethylammonium bromide (BDH Chem. Ltd.), redissolving the precipitate in 0.5 M Na_2SO_4, and dialyzing extensively against distilled water. The material was then deproteinized by the phenol method and dialyzed.[34] The final product had a reduced viscosity of 490 cc per g, an emulsifying activity of 150 units per mg, an ester content of 0.5 μ moles per mg, and a protein content of less than 0.5%. This emulsan preparation was dissolved in PUM buffer (2 mg/ml) and was sterilized by autoclave prior to its addition in the growth experiments.

RESULTS

Bacterial Adherence to Hydrocarbons. The basic assay for bacterial adherence to hydrocarbons is illustrated in

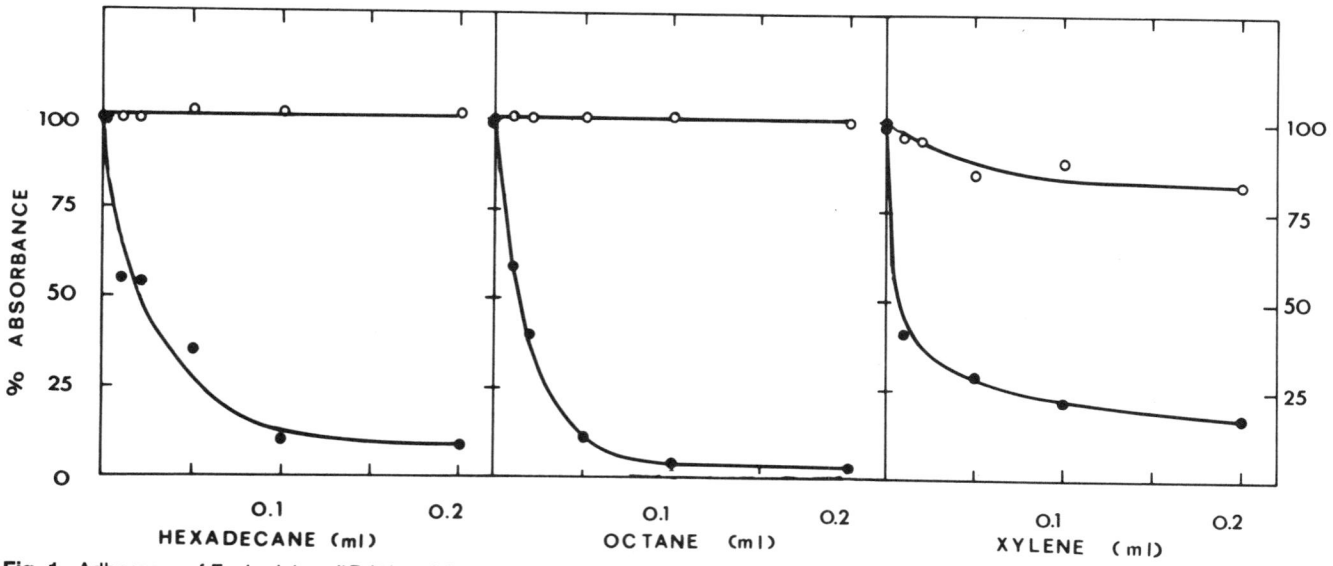

Fig. 1. Adherence of *Eschericia coli* B (○) and *Acinetobacter calcoaceticus* RAG-1 (●) to hydrocarbons. Cells were harvested at the end of the exponential growth phase, washed twice in PUM buffer, and then resuspended to an initial A_{400} of 1.2–1.4. The adherence assay was performed as described in "Materials and Methods." The ordinate is the percentage of initial absorbance of the cell suspension

Figure 1. The assay was performed with varying volumes of three test hydrocarbons: n-hexadecane, n-octane, and p-xylene. Following phase separation the turbidity of the lower aqueous phase was compared to that of the initial suspension. When the assay was performed with cells having no affinity for the test hydrocarbons (e.g., *E. coli*), the hydrocarbon droplets rose and coalesced soon after mixing and no significant changes were observed in the turbidity of the lower aqueous phase. However, when adherent cells were similarly tested (e.g., *A. calcoaceticus* RAG-1), a creamy upper layer was obtained following mixing. Microscopic observation of this layer revealed an oil-in-water emulsion consisting of hydrocarbon droplets, each covered by a layer of bacteria. The extraction of cells into this emulsion layer was accompanied by a corresponding decrease in turbidity of the lower aqueous phase. The fraction of adherent cells was determined by comparing the optical densities of the lower aqueous phase before and after mixing. Bacterial adherence was a function of volume of hydrocarbon added. The cell-coated hydrocarbon droplets in the upper layer remained stable for a period of days. Addition of 5% isopropanol resulted in immediate droplet coalescence and the concommitant release of extracted cells into the aqueous phase, thereby demonstrating that the bacteria had partitioned to the hydrocarbon-water interface.

Tables 1 and 2 summarize a number of experiments in which cell surface hydrophobicity of a variety of bacteria was measured by adherence to hexadecane. The majority of laboratory strains examined exhibited low affinity toward the test hydrocarbon. Among the bacterial strains exhibiting high affinity for hexadecane were hydrocarbon-degrading *Acinetobacter calcoaceticus* strains RAG-1, HO1-N, B5W str 71, and CDC 7828; and bacteria that do not metabolize hydrocarbons: *Serratia marcescens*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.

Pseudomonas aeruginosa (which can utilize hexadecane as the sole carbon and energy source) exhibited no significant affinity toward any of the test hydrocarbons when grown to late exponential phase in nutrient broth (Figure 2). Since adherence in this organism might have required the presence of hydrocarbons during growth, *P. aeruginosa* cells were also assayed after growth on hexadecane (Figure 2). The affinity of alkane-grown *P. aeruginosa* toward hexadecane increased somewhat, e.g., 32% of such cells were removed from the aqueous phase in the presence of 0.2 ml hexadecane. In contrast, *A. calcoaceticus* RAG-1 exhibited much higher affinity when grown on hexadecane; 93% of the cells could be removed by as little as 0.05 ml hexadecane.

Role of Adherence in the Growth of A. calcoaceticus *RAG-1 on Hexadecane.* *Acinetobacter calcoaceticus* RAG-1 adheres avidly to hydrocarbons.[35,36,37] A spontaneous mutant of RAG-1, deficient in its ability to adhere to hydrocarbons, was selected by enriching for cells that did not adhere to octane. This mutant, referred to as MR-481, was unable to adhere to any of the three test hydrocarbons, even when grown under conditions for which RAG-1 adherence was optimal (Figure 3).

Mutant MR-481 resembled the wild type in the following characteristics: (*i*) colonial and cell morphology,

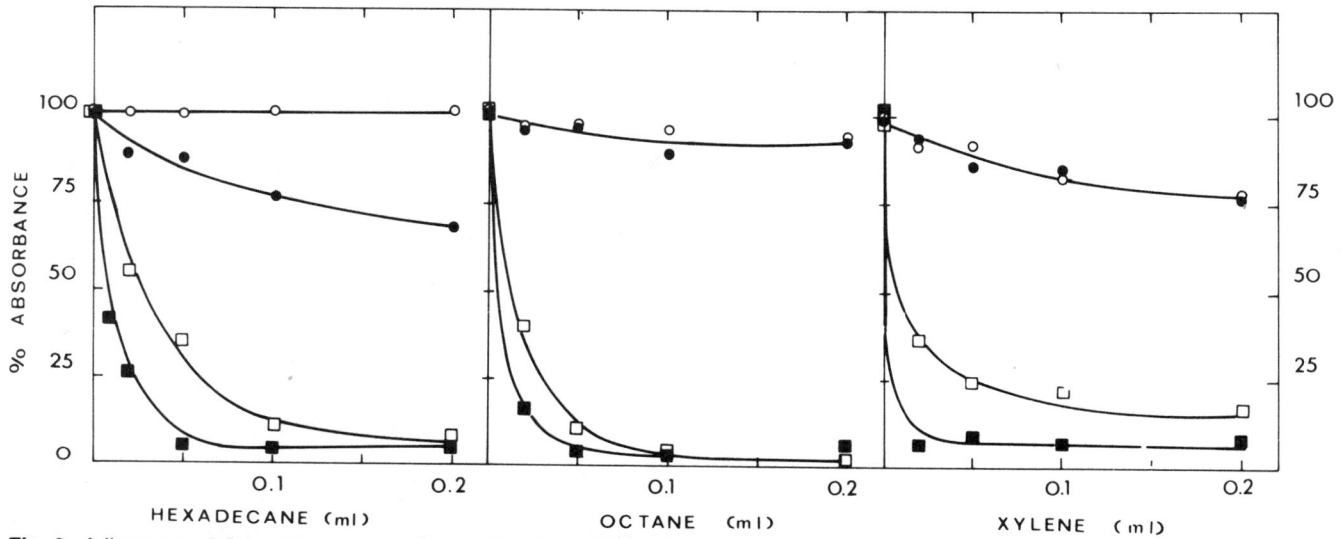

Fig. 2. Adherence of *Pseudomonas aeruginosa* PAS 279 and *Acinetobacter calcoaceticus* RAG-1 to hydrocarbons. The experiment was performed as described in Figure 1 with cells harvested at the end of the exponential growth phase: *P. aeruginosa*, NB medium (○), P-hexadecane medium (●); *A. calcoaceticus*, NB medium (□), hexadecane medium (■)

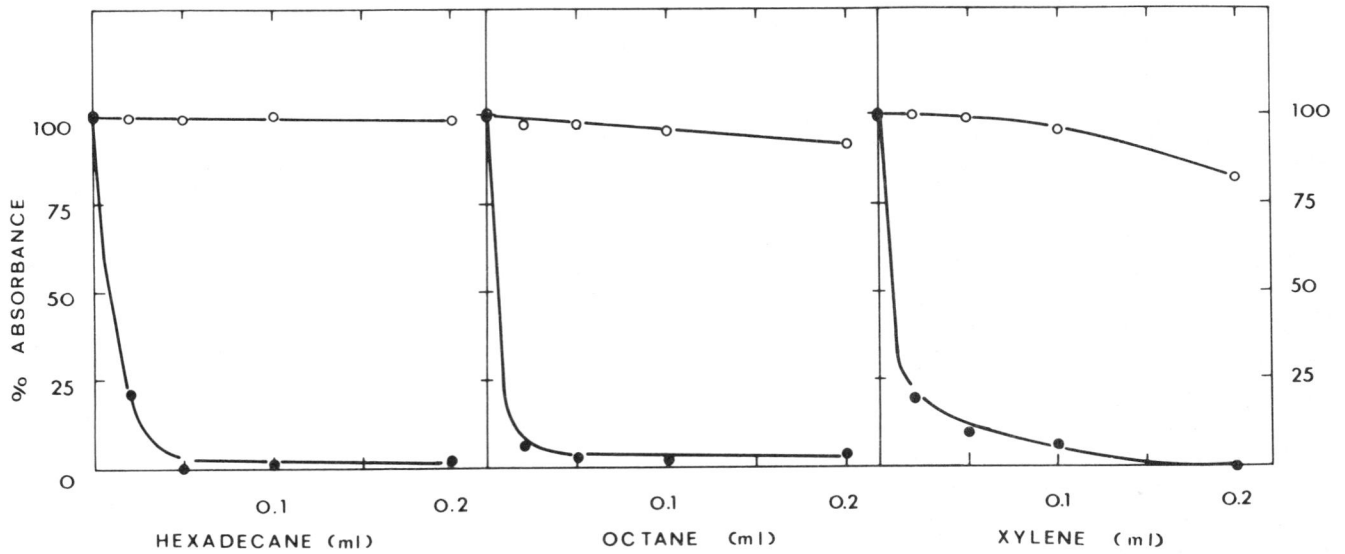

Fig. 3. Adherence of RAG-1 and MR-481 to hydrocarbons. Stationary phase cells were harvested from brain heart infusion broth, washed twice, and resuspended in PUM buffer to an A_{400} = 1.45 to 1.50. To 1.2 ml of suspended RAG-1 (●) or MR-481 (○) cells were added various volumes of test hydrocarbon, and the mixtures were vortexed for 120 sec. Following phase separation the turbidity of the lower aqueous phase was measured. The ordinate is the percent of initial absorbance of the cell suspension

(*ii*) sensitivity to two bacteriophages, ap2 and ap3, that are specific for *A. calcoaceticus* RAG-1 strains,[38] (*iii*) production of extracellular emulsifying activity during growth on ethanol as the sole carbon and energy source,[39] and (*iv*) doubling time on brain heart infusion broth (38 min at 30°C with aeration).

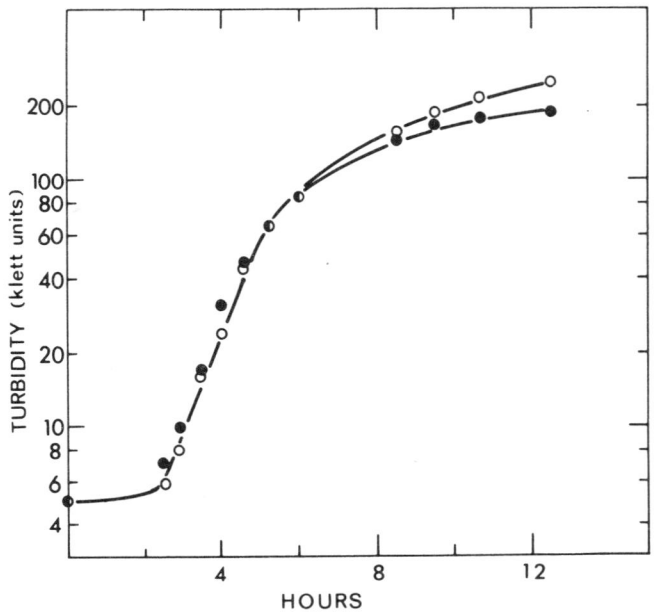

Fig. 4. Growth of RAG-1 and MR-481 in an acetate medium. Stationary phase cells were harvested from brain heart infusion broth, washed twice, and resuspended in PUM buffer supplemented with 0.2% sodium acetate. RAG-1 (●) and MR-481 (○) cells were inoculated into a 4.2-ml acetate medium in Klett tubes to an initial turbidity of 5 Klett units

In all growth experiments, inocula were prepared in the same manner as was described for measuring adherence in Figure 3. In a defined medium with sodium acetate as the soluble carbon and energy source, RAG-1 and MR-481 showed similar growth kinetics (Figure 4). Following a 2-hr lag, both strains grew with a doubling time of 48 min.

Using hexadecane as the sole source of carbon and energy (and under limited dispersion conditions), RAG-1 grew, whereas the nonadherent mutant MR-481 failed to grow for at least 54 hr (Figure 5). Growth of RAG-1 was accompanied by breaking the upper hydrocarbon layer into droplets. Microscopic examination of the upper phase indicated that these droplets were covered by patches of adhering cells. Hydrocarbon droplets were not observed in the aqueous phase under these conditions; thus, the observed turbidity of the lower aqueous phase was due to unbound cells. The values presented for growth of RAG-1 on hexadecane should be considered minimal since attached bacteria were not measured. In the corresponding tubes inoculated with mutant MR-481, neither growth nor substantial breakage of the hexadecane layer into droplets was observed (Figure 6).

Effect of Emulsan on the Growth of MR-481 on Hexadecane. *A. calcoaceticus* RAG-1 produces a potent extracellular emulsifying agent, referred to as emulsan.[40,41,42,43] It was of interest to examine the effect of a highly purified preparation of emulsan (see "Materials and

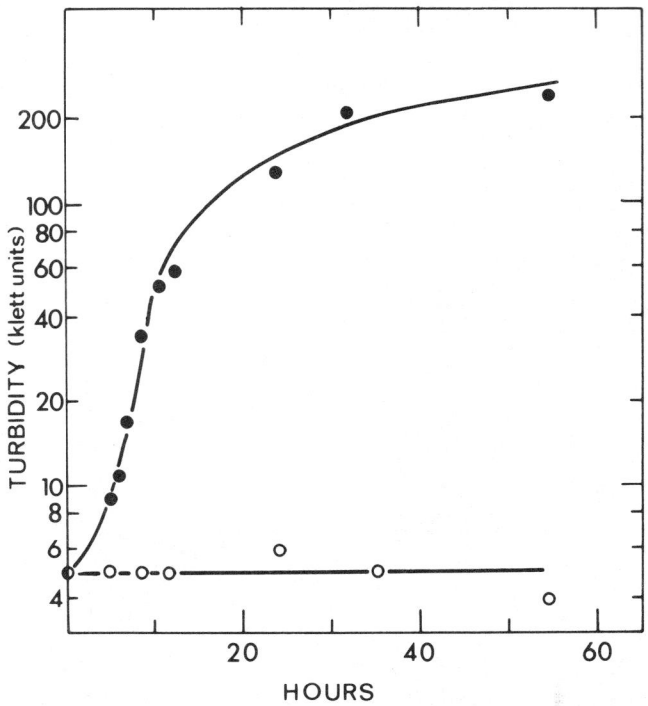

Fig. 5. Growth of RAG-1 and MR-481 in a hexadecane medium. Klett tubes containing 4.2 ml PUM buffer were inoculated with either RAG-1 (●) or MR-481 (○) to an initial turbidity of 5 Klett units. Tubes were overlayed with 0.5 ml hexadecane and were incubated as described in "Materials and Methods"

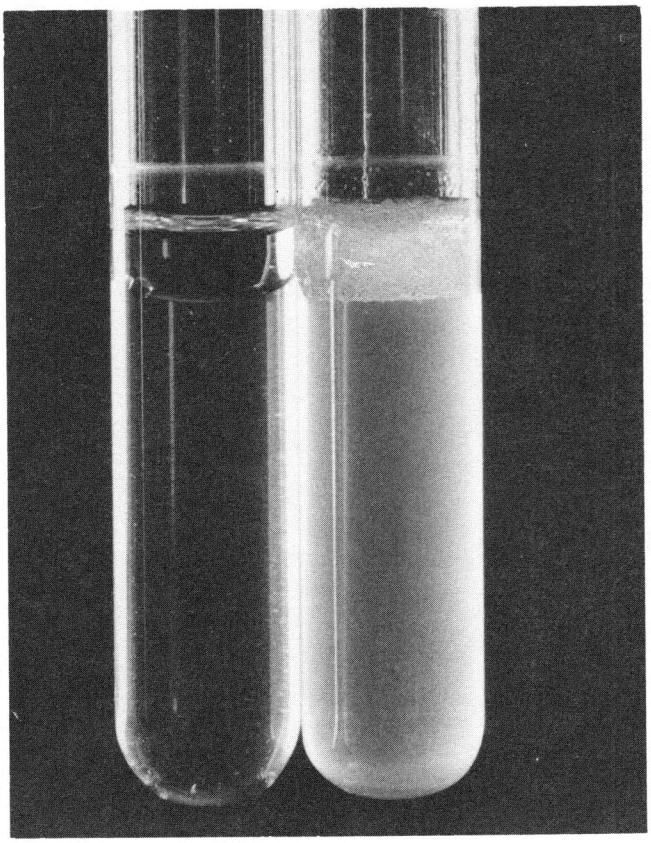

Fig. 6. Growth of MR-481 (left) and RAG-1 (right) on hexadecane medium. Tubes were photographed following 48 hr of incubation under the conditions described in Figure 3

Methods") on the growth on hexadecane of RAG-1 and the nonadherent mutant MR-481 (Figure 7). In the absence of hexadecane, emulsan did not serve as a carbon and energy source for either RAG-1 or MR-481. However, emulsan did enable growth of MR-481 on hexadecane. Growth of MR-481 in the presence of both emulsan and hexadecane began after about 6 hr, whereas neither component alone supported growth for 54 hr. At the weight ratio of hexadecane-emulsan used (1,925:1), the hexadecane droplets formed were too large to disperse into the aqueous phase and thus did not interfere with turbidity measurements. Emulsan had no significant effect on the growth of RAG-1 on hexadecane under these conditions.

Growth of MR-481 on hexadecane was also induced if the emulsan was added after several hours of incubation (Figure 8). In each case of emulsan addition, the resultant breakage of the hexadecane layer into droplets was followed by subsequent growth of the mutant cells. The time required for the commencement of growth following emulsan addition appeared to increase with the length of incubation prior to adding the bioemulsifier.

Growth of MR-481 on hexadecane in the presence of emulsan was not the result of genotypic nor phenotypic reversion of MR-481 from nonadherent to adherent cells. As shown in Table 3, there was no significant increase in the adherence of MR-481 grown on hexadecane in the presence of emulsan compared to the initial inoculum grown in a rich medium (Figure 3).

RAG-1 cells that were harvested and washed following growth on hexadecane both in the presence and absence of emulsan exhibited high affinity toward hexadecane (Table 3). Thus, the appearance of free RAG-1 cells in the lower phase during growth on hexadecane did not result from a loss in their adherent properties.

DISCUSSION

Mudd and Mudd reported over fifty years ago that certain microorganisms tend to concentrate at oil-water interfaces.[44] Although this observation has been corroborated frequently during studies on the growth of microorganisms on liquid hydrocarbons, there has been no direct evidence that adherence is an essential aspect of microbial growth on hydrocarbons.[45,46,47,48,49,50] Certain investigators have argued

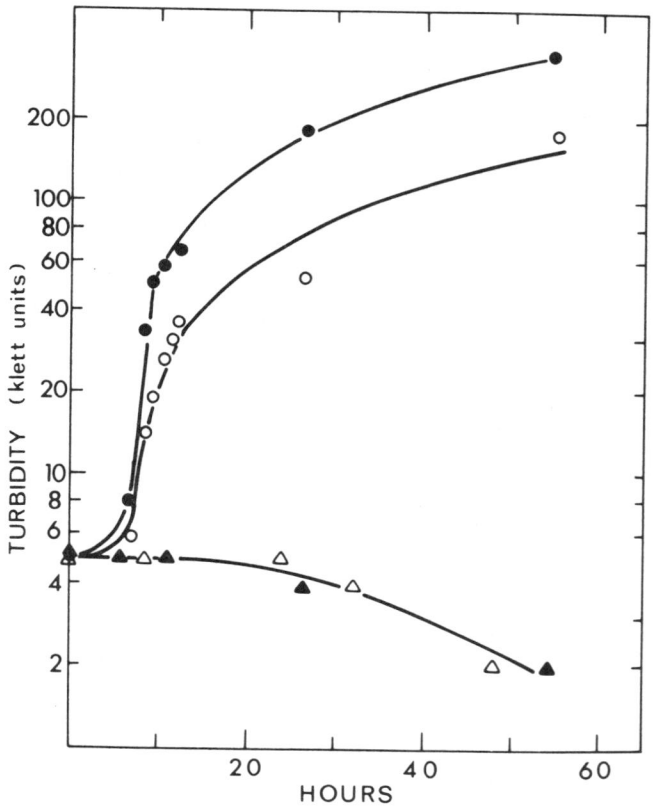

Fig. 7. Effect of emulsan on the growth of MR-481 on hexadecane. Inocula preparation and incubation conditions were as described in Figure 3. RAG-1 (●) and MR-481 (○) were grown on a hexadecane medium in the presence of 50 μg/ml emulsan. RAG-1 (▲) and MR-481 (△) controls were grown in PUM buffer supplemented with 50 μg/ml emulsan in the absence of hexadecane

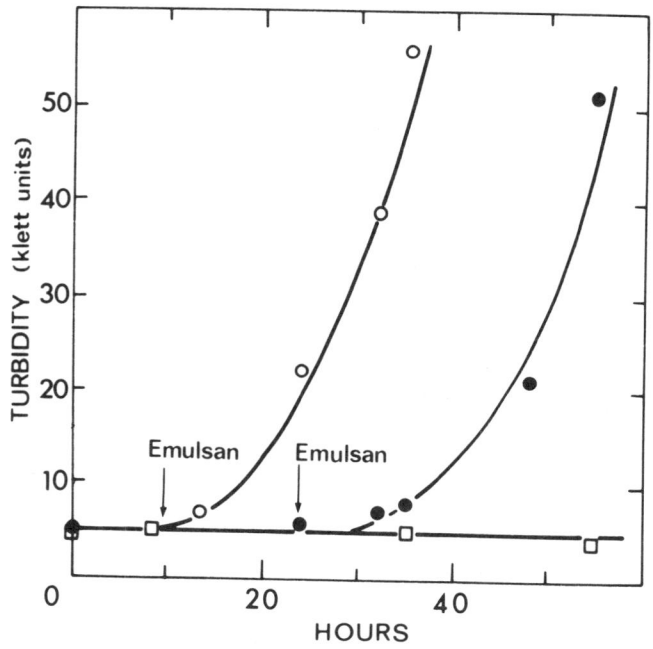

Fig. 8. Induction of growth of MR-481 on hexadecane by emulsan addition. Inocula preparation and incubation conditions were as described in Figure 3. Emulsan was added to a final concentration of 50 μg/ml at 10 hr (○) or 24 hr (●). Tubes containing no emulsan served as controls (□)

Table 3. Adherence of RAG-1 and MR-481 following growth on hexadecane.

Strain	Turbidity (A_{400})[a]		% Adherence
	Initial	Final	
RAG-1[b]	1.42	0.09	94
RAG-1[c]	1.40	0.05	96
MR-481[c]	1.43	1.36	4.9

[a]Cells were washed twice and resuspended in PUM buffer to initial turbidities as indicated. Hexadecane (0.2 ml) was added to 1.2 ml of cell suspension and the mixture was vortexed for 120 sec. Following phase separation, the turbidity of the aqueous phase was measured.

[b]RAG-1 cells were harvested following 54 hr growth on a hexadecane medium, washed twice, and resuspended in PUM buffer.

[c]RAG-1 and MR-481 were harvested following 54 hr growth on a hexadecane medium in the presence of 50 μg/ml emulsan, washed twice, and resuspended in PUM buffer.

that microbial growth on hydrocarbons is due to emulsification of the water-insoluble substrate rather than by direct contact between cells and hydrocarbon droplets.[51,52,53] *A. calcoaceticus* RAG-1 is an interesting microorganism in this regard because it adheres avidly to hydrocarbons and produces an extracellular emulsifying agent, emulsan, that has been extensively characterized.[54,55,56,57,58,59,60]

Isolation of a mutant strain of *A. calcoaceticus* RAG-1 deficient in its ability to adhere to hydrocarbon enabled an examination of the role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane. The nonadherent mutant strain, MR-481, failed to grow on hexadecane under the moderate agitation conditions that supported growth of wild-type cells. Two trivial explanations for the inability of MR-481 to grow on hexadecane — insufficient oxygen and a faulty hydrocarbon-metabolizing enzyme system — were excluded by the following data: (*i*) MR-481 and RAG-1 cells grew with similar kinetics on an acetate medium under conditions identical to those employed for growth on hexadecane; (*ii*) MR-481 was able to grow on a hexadecane medium following addition of an emulsifier; and (*iii*) MR-481 cells were able to grow on a solid medium supplemented with hexadecane vapors as the sole carbon and energy source. We conclude from these data that adherence is a

prerequisite for growth of RAG-1 on liquid hexadecane in the absence of strong mechanical agitation or emulsification of substrate.

Both RAG-1 and MR-481 produce a potent extracellular emulsifying agent that enables growth of nonadhering MR-481 cells on hexadecane. The observation that MR-481 does not grow on hexadecane even though it produces normal amounts of emulsan can readily be explained by considering emulsification as a cell-density dependent phenomenon.[61,62,63] At the low inoculum employed (5×10^7 cells/ml), the concentration of emulsan that the cells produced was too low to emulsify the hydrocarbon and permit growth.

These studies suggest that adherence is a prerequisite for growth on liquid hydrocarbon under two conditions: low cell density and limited emulsification. The ability of MR-481 to grow on hexadecane following emulsan addition demonstrates that the presence of extracellular emulsifying agents can enable growth of nonadherent bacteria on hydrocarbons. The poor adherence of *P. aeruginosa* PAS 279 to hydrocarbon (Figure 2) suggests that its growth, similar to that of MR-481, depends on dispersion of the hydrocarbon phase by extracellular emulsification or vigorous mechanical agitation. In most natural environments conditions of low cell density and limited emulsification prevail; thus, the growth of poorly adhering Pseudomonas strains in shake flasks and fermentors carried out in the presence of extraneous emulsifiers or vigorous agitation probably does not reflect growth in the open environment, where emulsifiers and dispersed hydrocarbons would tend to diffuse away from the cell.[64,65,66,67]

The adherence to hydrocarbon of bacteria that do not metabolize hydrocarbons (Tables 1 and 2) can be explained in terms of general cell surface hydrophobicity.[68,69] *S. pyogenes*, for example, has been reported to adhere to buccal epithelial cells through hydrophobic interactions.[70] Its adherence to hydrocarbons may thus be mediated by such interactions. Similarly, hydrophobic cell surface components that enable *Serratia* to partition at the air-water interface may also promote their adherence to hydrocarbons as observed in this study.[71]

Bacterial hydrophobicity has been cited as an important factor in several biological phenomena, including bacterial pathogenicity,[72] and in adherence and growth of bacteria on various hydrophobic surfaces.[73,74,75,76] Adherence to hydrocarbons provides a simple quantitative technique for measuring cell surface hydrophobicity and can be used for the selection and isolation of nonadhering mutants. Adherence of RAG-1 to hydrocarbons may reflect a general phenomenon in microbiology: a biological advantage is conferred on microbial cells that can escape by means of hydrophobic surface components from the bulk aqueous phase to an interface that provides nutrition and enables growth.

SUMMARY

A simple, quantitative assay method was used to measure the adherence of a variety of bacteria to liquid hydrocarbons. Bacterial species differed greatly in their ability to adhere to hydrocarbons; even within the same species, *Acinetobacter calcoaceticus*, different strains varied greatly in their cell surface hydrophobicity. No direct correlation was found between adherence to hydrocarbons and the ability to metabolize hydrocarbons. For example, *Staphylococcus aureus*, a bacterium incapable of degrading hydrocarbons, adhered strongly to all test hydrocarbons, whereas *Pseudomonas aeruginosa* adhered poorly to hydrocarbons, despite its ability to grow on hydrocarbons under laboratory conditions.

The high affinity of *Acinetobacter calcoaceticus* RAG-1 towards liquid hydrocarbons enabled the isolation of a spontaneous nonadherent mutant, MR-481. Strain MR-481 exhibited no significant affinity toward the three test hydrocarbons yet resembled the wild type in many properties, including production of the extracellular emulsifying agent, emulsan. In order to study more closely the role of adherence in growth on hydrocarbons, RAG-1 and MR-481 were compared for growth on hexadecane under conditions of limited agitation and at low initial cell density. Adherent RAG-1 cells were able to grow rapidly under these conditions, whereas nonadherent MR-481 cells failed to grow for at least 54 hr. However, addition of emulsan either initially or at various times after inoculation enabled the nonadherent MR-481 cells to grow on hexadecane. Growth was not the result of reversion of MR-481 from nonadherent to adherent cells.

The data demonstrate that adherence is a crucial factor in the growth of *A. calcoaceticus* RAG-1 on hexadecane in the absence of extracellular emulsification of the substrate.

ACKNOWLEDGMENTS

We would like to thank R. Martinez for valuable advice during the performance of these experiments and N. Kaplan for critical evaluation of the manuscript.

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18 GLYCOPROTEIN SURFACTANTS STABILIZE LONG-LIVED GAS MICROBUBBLES IN THE ENVIRONMENT

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The existence of long-lived gas microcavities or microbubbles in ordinary water and other aqueous liquids (e.g., body fluids) has been postulated or demonstrated by numerous investigators over the last four decades.[1-24] However, there is far less agreement in the literature as to the predominant physicochemical mechanism by which such microbubbles are stabilized. A detailed knowledge of this physicochemical stabilization mechanism in aqueous media is of practical importance to fields as varied as offshore drilling, naval hydrodynamics, hydraulic and ocean engineering, meteorology, chemical oceanography, underwater acoustics, surface chemistry, marine biology, the continual medical problem of decompression sickness, and enhanced oil recovery. Recent experimental work using carbohydrate (agarose) gels derived from marine algae has provided much evidence that largely nonionic or hydrophobic surfactants surround and stabilize the long-lived gas microbubbles present in such aqueous gels.[25-30] The concept of surfactant-stabilized microbubbles is in complete accord with the fact that surface-active substances in aqueous liquids, usually detected as trace organic contaminants, have long been known to affect both the cavitation threshold and the stability of bubbles formed in these liquids.[31-43] Even in the open sea, which is known to contain dissolved and nonliving particulate organic matter and specifically surface-active substances, an extensive microbubble population has been detected by various workers.[44-68] Consequently, it appeared likely that the naturally occurring, largely hydrophobic surfactants stabilizing long-lived gas microbubbles in agarose gels were present not only in marine algae (from which the gels were derived) but probably also in sea water and other environmental sources as well.[69-72] Accordingly, a systematic screening of a wide variety of natural substances revealed that (filtered) aqueous extracts of ordinary forest soil contained unusually high concentrations of gas microbubbles. This finding was reassuring and consistent with the fact that humic substances, similar (although not identical) to those occurring in soils, have been found to be widespread in sea water by various investigators.[73-77] An ongoing experimental program was thereafter expanded to characterize further and compare chemically the surfactants stabilizing long-lived microbubbles in both agarose gels and forest soil extracts.

INITIAL EXPERIMENTAL WORK WITH AGAROSE GELS

Over the last decade, bubble formation in gelatin has provided a useful model for studying the etiology of decompression sickness in humans.[78,79] From these and other studies, it has become clear that bubbles originate in water and probably also in humans as long-lived gas microbubbles, which must be held intact by surface-active films that are gas permeable.[80] In order to study the physical properties of the surfactant monolayer films surrounding gas microbubbles, a different gel method was developed that utilizes agarose as the gelling substance.[81,82] Unlike gelatin, which is composed of a complex mixture of charged peptide chains, agarose is an uncharged, relatively inert, homogeneous polysaccharide that is commercially available in highly purified form.[83,84] Consequently, agarose gels make well-controlled, surface-chemical studies of bubble formation possible.

The pressure vessel, glass counting chambers, and their Plexiglas holder used in the experiments reviewed below are all similar to those described elsewhere.[85,86] As viewed through the Plexiglas wall of the pressure vessel, the inside

involved in methanogenic hydrocarbon decomposition, and this may be the case in nitrate-reducing cultures as well. Nevertheless, it is clear that these populations are able to oxidize and utilize at least some of the components of crude oil in the absence of molecular oxygen. Whether, in practical terms, it would be possible to design a microbially enhanced oil recovery system — dependent on its ability to grow anaerobically on crude oil if the population were a consortium of different bacterial types — remains to be seen. One particular difficulty with doing so might arise from the need to use temperature- and pressure-tolerant strains in the deeper reservoirs. Each member of the consortium would itself need to withstand the high ambient temperature and pressure. Alternatively, it may ultimately prove possible to characterize both the biochemistry of the anaerobic processes and their genetic determinants so that all of the required reactions might be combined into a single strain, able simultaneously to use oil anaerobically in anoxic cultures and to tolerate high temperature and pressure.

The environmental conditions in the deep reservoirs of the North Sea are unfavorable for microbial growth. Their depth results in temperatures of about 90°C and above and pressures approaching 300 atm. Water flooding and pressure maintenance would, of course, use sea water, and any proposed microbial inoculum would need to function satisfactorily at such a salinity. The immediate objective is to inquire whether this combination of factors might not exclude bacterial systems altogether. While some of the bacterial cultures able to grow anaerobically on crude oil will do so in the presence of sea water, the main attention has been directed to the effects of high temperature on bacterial growth.

The usually accepted limit for growth under laboratory conditions is 86°C, although growth can occur up to about 100°C in the boiling water of natural hot springs, and there are reports that this has been achieved in one laboratory.[1] The possibility of raising the upper temperature limit observed for a freshly isolated bacterial strain is interesting. Presumably growth becomes impossible at some temperature because of the thermal instability on one or more cellular components. If these were proteins, the implication would be that the rate of synthesis could not be raised high enough to compensate for the rate of thermal denaturation. Such a hurdle might be overcome in two ways: (*i*) by allowing cells to adjust by a series of adaptive changes to an increased level of derepression of the synthesis of the limiting component, or (*ii*) by mutational change to increase its thermal stability. Both are worth exploring. If the temperature limitation imposed by the most temperature-sensitive factor could be increased by a few degrees, the maximum growth temperature of the organism would presumably increase until the next thermostability obstacle was encountered. It might prove possible to circumvent this by repeating the adaptive or mutational change and so progress upward in a series of steps until too many individual limitations, all acting together, prevented any further rise in growth temperature.

This might be regarded as being comparable to the progressive adaptations exhibited by bacteria grown in the presence of trifluoroleucine or increasing concentrations of deuterium oxide.[2,3] Gradually increasing the concentrations of either of these materials enables the cells to grow at levels that were totally inhibitory on first contact. The adaptations were, however, unstable; bacteria adapted to grow at almost 100% D_2O totally lost their ability to do so after no more than two generations of growth again in H_2O, even though one-quarter of their nonexchangeable hydrogen atoms would have been deuterated.

One of the many problems of developing microbiological populations functional in situ in the deep reservoirs is the likely interactive effect of high pressure, temperature, and salinity. Little published data extend into the region of interest for enhanced oil recovery. The genetic control of tolerance to these external factors, while of great practical significance, is virtually unexplored; but there is some evidence to suggest that tolerance may be controlled genetically in a manner simpler than might at first seem to be the case.[4,5] This raises again the exciting prospect of using gene manipulation to combine into a single bacterial strain the properties necessary to allow anaerobic growth on crude oil and those that permit growth to occur in the downhole conditions of the deep reservoirs.

ACKNOWLEDGMENTS

Part of this work has been carried out under contract to the U.K. Department of Energy.

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21 CELL-SURFACE HYDROPHOBICITY AND ADHERENCE TO SURFACES WITH SPECIAL REFERENCE TO ORAL BACTERIA

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Cell surface hydrophobicity is an important factor in the adherence and subsequent proliferation of microorganisms on solid surfaces and at interfaces.[1] Hydrophobic interactions play a role in the adherence of bacteria to nonwettable plastics,[2] phagocytes and buccal epithelial cells,[3,4] and in the partitioning of cells at liquid-hydrocarbon and liquid-air interfaces.[5-12] Adherence of petroleum-degrading bacteria to their hydrophobic substrate is a prerequisite for growth under certain conditions.[13]

While the hydrophobic properties of many laboratory strains have been tested using a variety of qualitative and quantitative approaches,[14-18] few studies have undertaken the screening of fresh microbial isolates for cell surface hydrophobicity.[19,20,21] The adherent properties of bacterial isolates may change with prolonged laboratory cultivation.[22] To our knowledge, there have been no previous reports on the cell-surface hydrophobicity of bacteria adhering to the tooth surface. A quantitative technique for measuring bacterial hydrophobicity based on adherence to liquid hydrocarbons was recently described.[23,24] The simplicity of this method lends itself to the rapid screening of numerous microbial samples. In the present study the hydrophobic surface characteristics of 155 fresh microbial isolates obtained from the surfaces of extracted teeth and incubated matrices were examined. Over 70% of these isolates exhibited pronounced cell surface hydrophobicity, as determined by adherence to hexadecane.

MATERIALS AND METHODS

Isolation of microorganisms. Adherent microorganisms were isolated from four freshly extracted teeth, obtained from four patients, and from two Tofflemire stainless steel matrix bands cut to a size of 0.5 cm × 0.5 cm × 0.038 mm (Teledyne, Elk Grove Village, Illinois). The latter were sterilized and subsequently incubated within an acrylic bite plate in the mouth of a healthy, low-caries (low tooth decay) volunteer for 2 hr.[25]

Following their removal, teeth and matrices were washed extensively with 20 ml saline in sterile petri dishes, were transferred to test tubes containing 5 ml saline, and were vortexed for 2 min to remove loosely bound cells. Teeth and matrices were then placed in wells of sterile Costar dishes (Costar, Cambridge, Mass.) containing 5 ml saline and were exposed to sonic oscillation at 0°C for 15 sec at 20 watts power in a Braun Labtronic 1510 sonifier (standard probe). Appropriate dilutions were spread onto brain-heart infusion agar (Difco) and were incubated under aerobic conditions at 37°C. Colonies were chosen randomly from these plates for further study.

Incubation conditions. Following two passages on brain-heart infusion agar, isolated colonies were inoculated into test tubes containing 5 ml brain-heart infusion broth (Difco) and were grown for 48 hr at 37°C with slow reciprocal shaking. Cells were harvested, washed twice, and suspended in PUM buffer (22.2 g of $K_2HPO_4 \cdot 3H_20$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_20$, and distilled water to 1,000 ml; pH 7.1) to optical densities of 1.0–1.5 at 400 nm (Gilford model 240 spectrophotometer).

Adherence to hexadecane. The general method for measuring bacterial adherence to liquid hydrocarbons has been described previously.[26,27] To 1.2 ml of bacterial suspension, various volumes of hexadecane were added. After preincubation at 30°C, the two phases were vortexed for 120 sec on

a Thermolyne (Sybron) mixer. After the phases were allowed to separate, the aqueous phase was carefully removed and its light absorbance at 400 nm was measured. The proportion of adherent cells was determined by comparing the drop in absorbance following the mixing procedure with the absorbance of the suspension prior to the assay.

RESULTS

The four teeth used in the experiments are described in Table 1.

The number of adherent microorganisms released from the teeth and stainless steel matrices by sonic oscillation, as determined by colony-forming units on brain-heart infusion agar, is shown in Table 2. The two carious teeth, A and B, yielded approximately 70 times more cells than the noncarious teeth, C and D. The number of microorganisms isolated from each of the matrices was of the same order of magnitude as the number of cells released from noncarious teeth C and D. Different colonies appearing on the plates were selected at random, cloned, and subsequently examined for their ability to adhere to hexadecane.

To illustrate the technique, Figure 1 shows adherence of two of the strains as a function of increasing hexadecane volume. While adherent strain M2-14 (isolated from matrix 2) was increasingly removed from the aqueous phase by greater volumes of hexadecane, the turbidity of the suspension of cells of D-26 (from tooth D) was not significantly affected. Following the mixing procedure, adherent cells rose with the hydrocarbon droplets to form a stable upper cream, consisting of cell-coated droplets. In the case of nonadherent strains, the hydrocarbon droplets coalesced following the mixing procedure and little or no decrease in turbidity of the aqueous phase was observed. For the results presented in Table 2, cell suspensions were mixed in the presence of 0.2 ml hexadecane. Strains were scored adherent positive if more than 75% of the cells were removed to the aqueous-hydrocarbon interface following the assay procedure. With few exceptions the isolates examined were either totally adherent (over 90%) or not at all adherent (less than 10%).

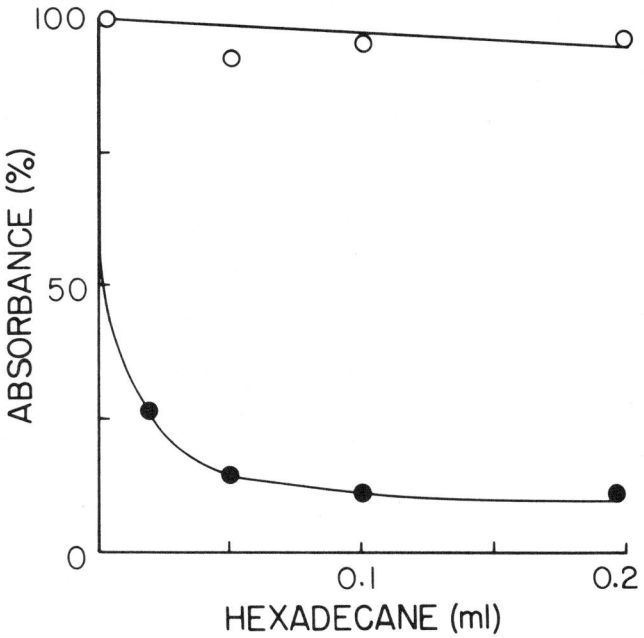

Fig. 1. Strains M2-14 (●) and D-26 (○) were compared for adherence to hexadecane. Initial optical absorbance: M2-14, 1.35; D-26, 1.23

Table 1. Description of extracted teeth used for isolating adherent bacteria.

Tooth[a]	*Condition*	*Reason for Extraction*
A	Untreated large caries	Caries
B	Untreated large caries	Caries
C	Intact	Pericorinitis
D	Root canal treatment and composite filling	Periodontitis

[a]Each of the four teeth was a third molar extracted from an adult male.

Table 2. Screening of oral bacteria for cell surface hydrophobicity.

Source of Microorganisms	*Total Colony-Forming Units*	*Isolates Examined*	*Isolates Adherent to Hexadecane*[a]
Tooth A	1.8×10^6	32	32
Tooth B	2.3×10^6	23	18
Tooth C	2.6×10^4	31	31
Tooth D	3.3×10^4	17	1
Matrix 1	1.2×10^4	29	13
Matrix 2	2.2×10^4	23	17

[a]Isolates were designated as adherent if more than 75% of the cells were removed following mixing in the presence of 0.2 ml hexadecane.

In teeth A and C, all of the isolates examined were hydrophobic. In tooth B, 78% of the isolates adhered to hexadecane, whereas only one of the 17 isolates from tooth D was adherent. A high frequency of hydrophobic bacteria was also isolated from the stainless-steel matrices that were incubated for only 2 hr in a bite plate in the mouth of a healthy volunteer (Table 2).

Although the hydrophobic bacteria isolated in the present report have not yet been classified, certain generalizations can be made: (*i*) all of the isolates obtained from tooth A were streptococci with similar cell and colony morphology;

(*ii*) the single isolate from tooth D that adhered to hexadecane was a gram-positive bacillus; (*iii*) hydrophobic isolates from teeth B and C and the two stainless-steel matrices comprised a wide variety of morphological types, including filamentous forms, coryneforms, bacilli, streptococcal and other coccal forms; and (*iv*) all the streptococci examined adhered to hexadecane.

DISCUSSION

Various studies have demonstrated that the tooth surface is normally covered by a hydrophobic film of organic matter termed "acquired pellicle."[28] While it has been postulated that the low surface energy imparted by this organic layer may act to impede bacterial attachment, it has also been suggested that hydrophobic interactions may actually participate in adherence of certain oral species to the tooth surface.[29,30,31]

A surprisingly high overall proportion of bacteria tested (72%) exhibited pronounced ability to adhere to the test hydrocarbon.

Bacterial isolates studied in this report probably represent a small fraction of the bacteria that adhere to and colonize the tooth surface, both because of the removal procedure (mild sonic oscillation) and cultivation conditions (aerobic, rich medium). Nevertheless, the diversity of adherent types found demonstrates for the first time that various species of hydrophobic bacteria can be isolated directly from the tooth surface. In addition, data obtained using the stainless-steel matrices demonstrate that hydrophobic microorganisms may become attached to surfaces following a brief incubation period in the oral cavity.

The demonstration of hydrophobic bacteria at the saliva-tooth interface raises a number of interesting questions that are now amenable to experimental examination: (*i*) Do hydrophobic microorganisms, similar to those isolated here, play a role in aiding or abetting formation of plaque, dental caries, and periodontal disease? (*ii*) Are similar mechanisms involved in adherence of these oral species to the tooth surface and to hexadecane? (*iii*) What intraoral factors influence the affinity of hydrophobic bacteria for the tooth-saliva interface? Further investigations into the possible role of hydrophobic interactions in mediating adherence of bacteria to the tooth surface are underway.

SUMMARY

Adherent bacteria were released from the surfaces of four freshly extracted teeth by mild sonic oscillation and were screened for cell surface hydrophobicity on the basis of their ability to adhere to hexadecane. Of the 103 tooth isolates examined, 82 adhered to the test hydrocarbon. Hydrophobic bacteria could similarly be isolated from stainless-steel dental matrix bands following brief incubation in the mouth of a volunteer; 30 to 52 isolates examined adhered to hexadecane. Among those strains that adhered to hexadecane, streptococci were the most frequent type isolated. Various other morphological types were also observed, including bacilli, coryneforms, and filamentous bacteria. The high overall proportion of hydrophobic bacteria found in this study (72%) suggests that cell surface hydrophobicity may play a role in adherence of certain oral species to the tooth surface.

ACKNOWLEDGMENTS

We would like to thank Yardena Mazor for excellent technical assistance and Yair Aharonowitz for critical reading of the manuscript.

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22 MICROBIAL DEGRADATION OF ATHABASCA BITUMEN

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The widespread decreased production of conventional oil has forced oil companies to seek other sources of petroleum. One of these, tar sand, is a mixture of approximately 85% sand and 15% bitumen with low percentages of water and clay minerals. Bitumen is an extremely viscous oil with a specific gravity of approximately 1. The origin of the tar sands is in dispute, but current evidence supports the view that these deposits represent the end product obtained from microbial degradation of a conventional crude oil.[1]

Figure 1 shows the tar sand deposits of Northern Alberta. There are estimated to be from 800 billion to 1.2 trillion barrels of oil locked up in these deposits.[2,3] Roughly 10% of this total is recoverable by surface mining technology. The remaining 90% is buried so deeply that some sort of in situ operation will be required to extract the oil. At present, there are two commercial-scale plants in operation, producing 60,000–120,000 bbl per day. Both of these plants use surface mining technology followed by hot-water treatment to separate the bitumen from the sand. Two other plants are in the late planning stage, one to use surface mining technology and the other to use an in situ method.

One of the main environmental concerns with both the hot-water process and in situ steam stimulation is that large quantities of bitumen-contaminated water are produced (roughly 2.5 bbl of water per barrel of oil). This water also contains clay minerals that will not settle, thus causing the water to be impounded for extremely long times before it can be recycled or returned to the environment. These large volumes of bitumen-contaminated water pose a potential threat to the environment. Microbial activity may have a role to play in purifying this water. Gemini Biochemical Research Ltd. has two projects in progress to study this activity. One project studies the use of microorganisms to clean up water produced by in situ plants.[4] The other, to be presented here, studies the microbial activity in the tailings ponds from surface mining plants.

The objectives of this project are:

1. To monitor the levels of dispersed bitumen in the ponds.
2. To fractionate the bitumen and monitor changes, if any, in these fractions with time.
3. To study the microbial populations of the ponds.
4. To select for organisms which can degrade the bitumen.
5. To study conditions required to maximize the degradation.

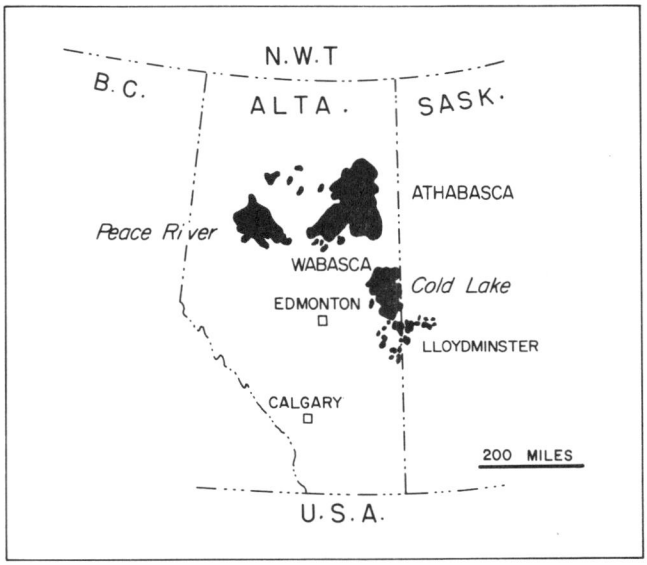

Fig. 1. Map of Alberta showing the location and extent of the oil sands deposits